

E' ribonucleotide (rA). (The biotin label is indicated via the encircled letter "B".) This primer is extended by *Taq* polymerase to yield a DNA product that contains a single embedded ribonucleotide. The resulting double-stranded DNA is immobilized on a streptavidin matrix and the unbiotinylated DNA strand is removed by washing with 0.2 N NaOH. After re-equilibrating the column with a buffered solution, the column is washed with the same solution with added 1 mM PbOAc. DNAs that undergo Pb²⁺-dependent self-cleavage are released from the column, collected in the eluant, and amplified by PCR. The PCR products are then used to initiate the next round of selective amplification.

At Page 16, line 20, please substitute the following paragraph for the previous version:

E2 Figure 3 illustrates the sequence alignment of individual variants isolated from the population after five rounds of selection. The fixed substrate domain is shown at the top, with the target riboadenylate identified via an inverted triangle (SEQ ID NO 13). Substrate nucleotides that are commonly involved in presumed base-pairing interactions are indicated by vertical bars. Sequences corresponding to the 50 initially-randomized nucleotides are aligned antiparallel to the substrate domain. All of the variants are 3'-terminated by the fixed sequence 5'-CGGTAAGCTTGGCAC-3' (not shown; SEQ ID NO 1). Nucleotides within the initially-randomized region that are presumed to form base pairs with the substrate domain are indicated on the right and left sides of the Figure; the putative base-pair-forming regions of the enzymatic DNA molecules are individually boxed in each sequence shown. Conserved regions are illustrated via the

two large, centrally-located boxes.

At Page 17, line 4, please substitute the following paragraph for the previous version:

Figures 4A and 4B illustrate DNA-catalyzed cleavage of an RNA phosphoester in an intermolecular reaction that proceeds with catalytic turnover. Figure 4A is a diagrammatic representation of the complex formed between the 19mer substrate (5'-TCACTATrAGGAAGAGATGG-3', SEQ ID NO 2) and 38mer DNA enzyme (5'-ACACATCTCTGAAGTAGCGCCGCGTATAGTGACGCTA-3', SEQ ID NO 3). The substrate contains a single adenosine ribonucleotide ("rA", adjacent to the arrow), flanked by deoxyribonucleotides. The synthetic DNA enzyme is a 38-nucleotide portion of the most frequently occurring variant shown in Figure 3. Highly-conserved nucleotides located within the putative catalytic domain are "boxed". As illustrated, one conserved sequence is "AGCG", while another is "CG" (reading in the 5'-3' direction).

At Page 18, line 9, please substitute the following paragraph for the previous version:

Figures 6A and 6B provide two-dimensional illustrations of a "progenitor" catalytic DNA molecule and one of several catalytic DNA molecules obtained via the selective amplification methods disclosed herein, respectively. Figure 6A illustrates an exemplary molecule from the starting pool, showing the overall configuration of the molecules represented by SEQ ID NO 133. As illustrated, various complementary nucleotides flank the random (N_{40}) (SEQ ID NO 143) region. Figure 6B is a diagrammatic representation of one of the Mg^{2+} -dependent catalytic DNA

E4
molecules (or "DNAzymes") (SEQ ID NO 123) generated via the within-described procedures. The location of the ribonucleotide in the substrate nucleic acid is indicated via the arrow in both Figs. 6A and 6B.

At Page 19, line 4, please substitute the following paragraph for the previous version:

E5
Figure 8 illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 (SEQ ID NO 134) and 10-23 (SEQ ID NO 136). Reaction conditions were as shown, namely, 10mM Mg^{2+} , pH 7.5, and 37°C. The DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3' (SEQ ID NO 135)) -- which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated (SEQ ID NO 135). For the 8-17 enzyme, the turnover rate was approximately 0.6 hr^{-1} ; for the 10-23 enzyme, the turnover rate was approximately 1 hr^{-1} . Noncomplementary pairings are indicated with a closed circle (●), whereas complementary pairings are indicated with a vertical line (|).

At Page 19, line 22, please substitute the following paragraph for the previous version:

E6
Figure 9 further illustrates the nucleotide sequences,

E⁶
cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 (SEQ ID NO 138) and 10-23 (SEQ ID NO 137). Reaction conditions were as shown, namely, 10mM Mg²⁺, pH 7.5, and 37°C. As in Figure 8, the DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3' (SEQ ID NO 135)) --which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 residue nos. 5-33 of SEQ ID NO 85, with "CTA" substituted for "TTG" at the 5' end is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated (SEQ ID NO 135). For the 8-17 enzyme, k_{obs} was approximately 0.002 min⁻¹; for the 10-23 enzyme, the value of k_{obs} was approximately 0.01 min⁻¹. Noncomplementary pairings are indicated with a closed circle (●), whereas complementary pairings are indicated with a vertical line (|).

At Page 20, line 7, please substitute the following paragraph for the previous version:

E⁷
Figure 10 illustrates a schematic showing the composition of the 8-17 (SEQ ID NO 120) and 10-23 (SEQ ID NO 121) catalytic motifs. The DNA enzyme (bottom strand) binds the RNA substrate (top strand) through complementary Watson-Crick pairing (vertical lines) between unspecified complementary nucleotides (horizontal lines). Cleavage occurs at the position indicated by the arrow, where R = A or G and Y = U or C.

At Page 59, line 26, please substitute the following

paragraph for the previous version:

Figure 3 illustrates the sequence alignment of individual variants isolated from the population after five rounds of selection. The fixed substrate domain (5'-GGGACGAATTCTAATACGACTCACTATrAGGAAGAGATGGCGAC-3' (SEQ ID NO:13), or 5'-GGGACGAATTCTAATACGACTCACTATNGGAAGAGATGGCGAC-3', where N represents adenosine ribonucleotide) (SEQ ID NO 13) is shown at the top, with the target riboadenylate identified with an inverted triangle. Substrate nucleotides that are commonly involved in presumed base-pairing interactions are indicated by a vertical bar. Sequences corresponding to the 50 initially-randomized nucleotides are aligned antiparallel to the substrate domain. All of the variants are 3'-terminated by the fixed sequence 5'-CGGTAAGCTTGGCAC-3' (SEQ ID NO 1) ("primer site"; not shown). Nucleotides within the initially-randomized region that are presumed to form base pairs with the substrate domain are indicated on the right and left sides of the Figure; the putative base-pair-forming (or substrate binding) regions of the enzymatic DNA molecules are individually boxed in each sequence shown. The highly-conserved nucleotides within the putative catalytic domain are illustrated in the two boxed columns.

At Page 61, line 28, please substitute the following paragraph for the previous version:

Synthetic DNAs and DNA analogs were purchased from Operon Technologies. The 19-nucleotide substrate, 5'-pTCACTATrAGGAAGAGATGG-3' (SEQ ID NO:7) (or 5'-pTCACTATNGGAAGAGATGG-3', wherein "N" represents adenosine

E⁹
ribonucleotide) (SEQ ID NO 7), was prepared by reverse-transcriptase catalyzed extension of 5'-pTCACTATrA-3' (SEQ ID NO:8) (or 5'-pTCACTATN-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 8), as previously described (Breaker et al, Biochemistry, 33:11980-11986, 1994), using the template 5'-CCATCTCTTCCTATAGTGAGTCCGGCTGCA-3' (SEQ ID NO 9). Primer 3, 5'-GGGACGAATTCTAATACGACTCACTATrA-3' (SEQ ID NO:6) (or 5'-GGGACGAATTCTAATACGACTCACTATN-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 6), was either 5'-labeled with [γ -³²P]ATP and T4 polynucleotide kinase (primer 3a) or 5'-thiophosphorylated with [γ -S]ATP and T4 polynucleotide kinase and subsequently biotinylated with N-iodoacetyl-N'-biotinylnhexylenediamine (primer 3b).

At Page 66, line 2, please substitute the following paragraph for the previous version:

E¹⁰
In designing the catalytic domain, we relied heavily on the composition of the most reactive variant, truncating by two nucleotides at the 5' end and 11 nucleotides at the 3' end. The 15 nucleotides that lay between the two template regions were left unchanged and a single nucleotide was inserted into the 3' template region to form a continuous stretch of nucleotides capable of forming base pairs with the substrate. The substrate was simplified to the sequence 5'-TCACTATrA \odot GGAAGAGATGG-3' (SEQ ID NO:12) (or 5'-TCACTATN \odot GGAAGAGATGG-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 12), where the underlined nucleotides correspond to the two regions involved in base pairing with the catalytic DNA molecule.

At Page 71, line 24, please substitute the following paragraph for the previous version:

E¹¹
Individual clones were isolated following the sixth round, and the nucleotide sequence of 24 of these clones was determined. All of the sequences began with: 5' GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA CA (SEQ ID NO 139) and ended with: CGG TAA GCT TGG CAC 3' (SEQ ID NO 1).

At Page 71, line 30, please substitute the following paragraph for the previous version:

E¹²
The segment in the middle, corresponding to TCTC N₄₀ GTGA (SEQ ID NO 140) in the starting pool, varied as follows:

At Page 73, line 16, please substitute the following paragraph for the previous version:

E¹³
The lead compound was re-randomized at 40 nucleotide positions (underlined), introducing mutations at a frequency of 15% (5% probability of each of the three possible base substitutions). The re-randomized population was subjected to seven additional rounds of *in vitro* selection. During the last four rounds, molecules that were reactive in the presence of 1 mM Pb²⁺ were removed from the population before the remainder were challenged to react in the presence of 1 mM Mg²⁺. Individual clones were isolated following the seventh round and the nucleotide sequence of 14 of these clones was determined. All of the sequences began with: 5' GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA CAT CTC (SEQ ID NO 141), and ended

E13
with: GTG ACG GTA AGC TTG GCA C 3' (SEQ ID NO 142).

At Page 73, line 31, please substitute the following paragraph for the previous version:

E14
The segment in the middle, corresponding to the 40 partially-randomized positions (N₄₀, SEQ ID NO 143), varied as follows:

At page 74, line 28, please substitute the following paragraph for the previous version:

E15
Figures 6A and 6B provide two-dimensional illustrations of a "progenitor" catalytic DNA molecule and one of several catalytic DNA molecules obtained via the selective amplification methods disclosed herein, respectively. Figure 6A illustrates an exemplary molecule from the starting pool, showing the overall configuration of the molecules represented by SEQ ID NO 23. As illustrated, various complementary nucleotides flank the random (N₄₀) (SEQ ID NO 143) region.

At Page 76, line 11, please substitute the following paragraph for the previous version:

E16
The initial library was generated by template-directed extension of 50 pmols of 5'-biotin-d(GGAAAAA)r(GUAACUAGAGAU)d(GGAAGAGATGGCGAC)-3' (SEQ ID NO 144) on 100 pmols of 5'-GTGCCAAGCTTACCG-N50-GTCGCCATCTCTTCC-3' (SEQ ID NO 4) (N = G, A, T or C), in a 50-ul reaction mixture containing 10 U ul⁻¹ Superscript II reverse transcriptase (RT; Gibco BRL), 3 mM MgCl₂, 75 mM KCl, 50 mM Tris*HCl (pH 8.3), and

E16
0.2 mM of each dNTP. A trace amount of [5'-32P]-labeled primer was included in the reaction mixture to allow extension efficiency to be monitored. All components except RT were combined, incubated at 65 C for 5 min, then cooled to 45 C over 10 min. RT was added and the mixture was incubated at 45 C for 45 min, then quenched by addition of Na2 EDTA. NaCl was added to a final concentration of 1 M and the extension products were immobilized by repeated passing through four streptavidin affinity columns (Genosys). The columns were washed with five 100-ul volumes of wash buffer (1 M NaCl, 50 mM Tris*HCl (pH 7.5), 0.1 mM Na2EDTA), followed by five 100-ul volumes of 0.1 N NaOH and five 100-ul volumes of wash buffer at 37 C, then eluted at 37 C over 1 hr with three 20-ul aliquots of reaction buffer (10 mM MgCl2, 1 M NaCl, 50 mM Tris*HCl (pH 7.5)). Eluted molecules were recovered and amplified by the polymerase chain reaction (PCR) using the primers 5'-biotin-GGAAGAGATGGCGAC-3' (SEQ ID NO 145) and 5'-GTGCCAAGCTTACCG-3' (SEQ ID NO 10). The PCR products were immobilized on streptavidin columns, as above, which were washed with five 100-ul volumes of wash buffer and eluted with 40 ul of 0.1 N NaOH to obtain the non-biotinylated strand. The isolated DNAs were ethanol precipitated and used as templates in a primer extension reaction to begin the next round of selection. Rounds 2-10 were carried out as above, except that the reaction scale was reduced five-fold during the extension step and two-fold during PCR.

At Page 78, line 12, please substitute the following paragraph for the previous version:

E17
Under the heading "Nucleotide Sequence" in each of Tables 2 and 3 is shown the portion of each identified clone that

E17
corresponds to the 50 nucleotides that were randomized in the starting pool (i.e., N₅₀ (SEQ ID NO 146)); thus, the entire nucleotide sequence of a given clone generally includes the nucleotide sequences preceding, following, and including the "N₅₀" (SEQ ID NO 146) segment, presuming the substrate sequence is attached and that self-cleavage has not occurred. For example, the entire sequence of a (non-self-cleaved) clone may generally comprise residue nos. 1-33 of SEQ ID NO 50, followed by the residues representing the randomized N₅₀ (SEQ ID NO 146) region, followed by residue nos. 84-98 of SEQ ID NO 50, or by residue nos. 1-34 of SEQ ID NO 51, followed by the residues representing the randomized N₅₀ (SEQ ID NO 146) region, followed by residue nos. 85-99 of SEQ ID NO 51. It is believed, however, that the N₅₀ (SEQ ID NO 146) (or N₄₀ (SEQ ID NO 143) region -- or a portion thereof -- of each clone is particularly important in determining the specificity and/or activity of a particular enzymatic DNA molecule. This is particularly evident in reactions in which the substrate and the DNAzyme are separate molecules (see, e.g., Figs. 8 and 9).

At Page 78, line 33, please substitute the following paragraph for the previous version:

E18
Clone numbers are designated as 8-x or 10-x for individuals obtained after the 8th or 10th rounds, respectively. SEQ ID NOS are also listed and correspond to the "N₅₀" (SEQ ID NO 146) region of each clone.

At Page 82, line 24, please substitute the following paragraph for the previous version:

E¹⁹
The self-cleavage activity of various clones was subsequently measured. Clones 8-5, 8-17, and 10-3 were found to cleave efficiently at the site 5' GUAACUAGAGAU 3' (SEQ ID NO 49), while clones 10-14, 10-19 and 10-27 were found to cleave efficiently at the site 5' GUAACUAGAGAU 3' (SEQ ID NO 49). When the RNA portion of the molecule was extended to the sequence 5' GGAAAAAGUAACUAGAGAUGGAAG 3' (SEQ ID NO 135), clones 8-17, 10-14, and 10-27 retained full activity, while clones 8-5, 10-3, and 10-19 showed diminished activity. Subsequently, clone 10-23 was found to exhibit a high level of activity in the self-cleavage reaction involving the extended RNA domain.

At Page 83, line 2, please substitute the following paragraph for the previous version:

E²⁰
It should also be noted, in the event one of skill in the relevant art does not appreciate same, that the nucleotide sequences preceding and following the "N₅₀" (SEQ ID NO 146) segments of the polynucleotide molecules engineered according to the teachings of the present invention disclosure, i.e, the substrate binding regions flanking the "N₅₀" (SEQ ID NO 146) region, may be altered in a variety of ways in order to generate enzymatic DNA molecules of particular specificities, such as by length, nucleotide sequence, type of nucleic acid, and the like. For example, while residue nos. 1-24 of SEQ ID NO 51 are described herein as RNA nucleotides, they may alternatively comprise DNA, RNA, or composites thereof. (Thus, for example, SEQ ID NO 51 could easily be altered so that nucleic acid residue nos. 1-7 would comprise DNA, residue nos. 8-19 would comprise RNA, residue nos. 20-99 would comprise DNA, and so on.) Similarly, the nucleotides following the "N₅₀" (SEQ ID NO 146)

E20
region may comprise RNA, DNA, or composites thereof. The length of the regions preceding and following the "N₅₀" (SEQ ID NO 146) (or "N₄₀" (SEQ ID NO 143) -- see Example 4) region(s) may also be varied, as disclosed herein. Further, sequences preceding and/or following N₅₀ (SEQ ID NO 146) or N₄₀ (SEQ ID NO 143) regions may be shortened, expanded, or deleted in their entirety.

At Page 84, line 11, please substitute the following paragraph for the previous version:

E21
Figure 8 illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23. Reaction conditions were as shown, namely, 10mM Mg²⁺, pH 7.5, and 37°C. The DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3' (SEQ ID NO 135) -- which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme, the turnover rate was approximately 0.6 hr⁻¹; for the 10-23 enzyme, the turnover rate was approximately 1 hr⁻¹.

At Page 84, line 26, please substitute the following paragraph for the previous version:

E22
As illustrated in Figure 8, the nucleotide sequence of the clone 8-17 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows:

E22
5'-CTTCCACCTTCCGAGCCGGACGAAGTTACTTTTT-3' (SEQ ID NO 134). In that same figure, the nucleotide sequence of the clone 10-23 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows: 5'-CTTTGGTTAGGCTAGCTACAACGATTTTCC-3' (SEQ ID NO 136).

At Page 85, line 2, please substitute the following paragraph for the previous version:

E23
Figure 9 further illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23. Reaction conditions were as shown, namely, 10mM Mg^{2+} , pH 7.5, and 37°C. As in Figure 8, the DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3' (SEQ ID NO 135)) --which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme, k_{obs} was approximately 0.002 min⁻¹; for the 10-23 enzyme, the value of k_{obs} was approximately 0.01 min⁻¹.

At Page 85, line 17, please substitute the following paragraph for the previous version:

E24
As illustrated in Figure 9, the nucleotide sequence of the clone 8-17 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows:

E24
5'-CCACCTTCCGAGCCGGACGAAGTTACT-3' (SEQ ID NO 138). In that same figure, the nucleotide sequence of the clone 10-23 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows: 5'-CTAGTTAGGCTAGCTACAACGATTTTTTC-3' (SEQ ID NO 137) (residue nos. 5-33 of SEQ ID NO 85, with "CTA" substituted for "TTG" at the 5' end).

At Page 88, line 17, please substitute the following paragraph for the previous version:

E25
The re-selections based on the 8-17 and 10-23 molecules involved six different lineages for each motif. Each lineage entailed 5-21 rounds of in vitro selection, differing with respect to the selection protocol and reaction times. All cleavage reactions were carried out in 2 mM MgCl₂, 150 mM NaCl, and 50 mM Tris*HCl (pH 7.5) at 37 C. Reaction times varied from 60 min in early rounds to 1 min in later rounds. Each starting pool of templates was based on a sequence complementary to the prototype, with fixed binding arms of seven nucleotides each and a catalytic core randomized to 25% degeneracy at each nucleotide position. For the 8-17 and 10-23 motifs, the templates had the sequence

5'-gtgccaagccttaccgagtaactTCG-TCCGGCTCGGRagatgggtcgtctgtccttccATCTCTAGTTACTTTTTTC- 3' (SEQ ID NO 124) and

5'-gttgccaagccttaccg-ggaaaaaTCGTTGTAGCTAGCCTaactaggtcgtctgtccttccATCTCTAGT TACTTTTTTC-3' (SEQ ID NO 125), respectively (PCR primer sites in lower case; substrate-binding arms underlined; randomized positions italicized). The primer used in the template-directed extensions had the sequence

5'-biotin-r(GGAAAAA-GUAACUAGAGAUGG)d(AAGAGATGGCGAC)-3' (SEQ ID NO 132). The PCR primers for the 8-17-based selections were

E25
5'-GTGCCAAGCTTACCGAGTAACT-3' (SEQ ID NO 147) and
5'-d(GGAAGGACAGACGACC-CATC)rU (SEQ ID NO 148) and for the
10-23-based selections were 5'-GTGCCAAGCTTACCGGGAAAAA-3' (SEQ ID
NO 127) and 5'-d(GGAAGGACAGACGACCTAGTT)rA (SEQ ID NO 149). The
PCR primers encompassed the binding arms, thus fixing these
sequences. One of the PCR primers in each set contained a
3'-terminal ribonucleotide, allowing isolation of the template
strand from the double-stranded PCR products by alkaline
hydrolysis of the non-template strand and subsequent purification
by polyacrylamide gel electrophoresis. A gel-based selection
scheme was employed in some of the lineages. In those cases, the
PCR primers were 5'-biotin-GTGCCAAGCTTACCG-3' (SEQ ID NO 150) and
5'-GAAAAAGTAACTAG-AGATGGAAGGACAGACGACC-3' (SEQ ID NO 129) and the
extension reactions were carried out on the solid support using
the primer 5'-r(GGAAAAAGUAACUAGAGAUGGAAG)-3' (SEQ ID NO 135). A
trace amount of [α -³²P]-dATP was included in the mixture to label
the extension products, which were eluted with alkali, purified
by denaturing polyacrylamide gel purification, and recovered by
electroelution. The molecules then were reacted and those that
underwent cleavage were isolated by gel electrophoresis.

In The Claims:

At page 141, line 2, please substitute the following claim 1
for the previously submitted claim 1:

E26
1. A catalytic DNA molecule having site-specific
endonuclease activity specific for a nucleotide sequence defining
a cleavage site in a preselected substrate nucleic acid sequence,
said molecule having first and second substrate binding
regions flanking a core region,